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Androgens regulate scarless repair of the endometrial "wound" in a mouse model of menstruation

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ABSTRACT: The human endometrium undergoes regular cycles of synchronous tissue shedding (wounding) and repair that occur during menstruation before estrogen-dependent regeneration. Endometrial repair is normally both rapid and scarless. Androgens regulate cutaneous wound healing, but their role in endometrial repair is unknown. We used a murine model of simulated menses; mice were treated with a single dose of the nonaromatizable androgen dihydrotestosterone (DHT; 200 μg/mouse) to coincide with initiation of tissue breakdown. DHT altered the duration of vaginal bleeding and delayed restoration of the luminal epithelium. Analysis of uterine mRNAs 24 h after administration of DHT identified significant changes in metalloproteinases (*Mmp3* and *-9*; $P < 0.01$), a snail family member (*Snai3*; $P < 0.001$), and osteopontin (*Spp1*; $P < 0.001$). Chromatin immunoprecipitation analysis identified putative androgen receptor (AR) binding sites in the proximal promoters of *Mmp9*, *Snai3*, and *Spp1*. Striking spatial and temporal changes in immunoexpression of matrix metalloproteinase (MMP) 3/9 and caspase 3 were detected after DHT treatment. These data represent a paradigm shift in our understanding of the role of androgens in endometrial repair and suggest that androgens may have direct impacts on endometrial tissue integrity. These studies provide evidence that the AR is a potential target for drug therapy to treat conditions associated with aberrant endometrial repair processes.—Cousins, F. L., Kirkwood, P. M., Murray, A. A., Collins, F., Gibson, D. A., Saunders, P. T. K. Androgens regulate scarless repair of the endometrial "wound" in a mouse model of menstruation. FASEB J. 30, 000–000 (2016). www.fasebj.org

KEY WORDS: mesenchyme · epithelium · SNAI3 · metalloproteinase · dihydrotestosterone

In women, the most important tissues for biosynthesis of androgens are the ovaries, adrenals, and fat. In adult women, 50% of the testosterone measured in blood is secreted by the ovaries and adrenals, with a small midcycle peak (1). The other 50% is derived from conversion of adrenal and ovarian precursors, such as androstenedione, dehydroepiandrosterone, and dehydroepiandrosterone sulfate. Testosterone can be further metabolized to both estradiol and the potent nonaromatizable androgen dihydrotestosterone (DHT) in tissues that express aromatase and 5-α reductase enzymes, respectively (1–3).

During the menstrual cycle, endometrial breakdown at the time of menses is the culmination of a tightly controlled cascade of biomolecular changes initiated by progesterone (P4) withdrawal (demise of the corpus luteum), which results in shedding of the upper functional layer of the endometrium. Detailed morphologic evaluation in women has revealed that breakdown and restoration of tissue homeostasis at the time of menstruation in normal women occurs rapidly, with epithelialization of the exposed basal stroma completed within 4 d of the first evidence of bleeding (4, 5). Piecemeal degradation and bleeding that give the surface of the endometrium the appearance of a wound has been documented with a hysteroscope introduced *via* the vagina and cervix (4). At the time of tissue breakdown, there is concurrent neoangiogenesis, stabilization of residual basal blood vessels, and rapid reepithelialization (4). The repair of the endometrium in women is coincident with the time in the cycle when estrogens and P4 are at their lowest (6).

Inbred mice do not spontaneously menstruate, but we (7) and others (8, 9) have used hormonal manipulation of ovariectomized mice combined with manual stimulation of the endometrium to induce decidualization to replicate the key features of the process in women, including synchronous shedding and repair and rapid reepithelialization.

ABBREVIATIONS: AR, androgen receptor; ARE, androgen response element; ChIP, chromatin immunoprecipitation; DHT, dihydrotestosterone; MMP, matrix metalloproteinase; P4, progesterone

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In addition, results in xenografts of human endometrial tissue maintained in nude mice (10) have complemented the studies in mice (9), showing that restoration of an intact endometrial epithelium is independent of the action of estrogens. These findings distinguish the menstrual phase from the subsequent proliferative phase, during which rising concentrations of follicle-derived estrogens play a crucial role in endometrial growth and regeneration, as well as in promoting P4 receptor expression [see Hewitt *et al.* (11)].

Recovery of tissue integrity during menstruation exhibits parallels with repair strategies documented in epidermal wound healing, including inflammation, fibroblast migration, reepithelialization, and tissue remodeling (12). Evidence from studies of epidermal wound healing has identified a role for androgens in this process. For example, studies in male rats have reported that blocking biosynthesis of DHT accelerates the reepithelialization of incisional and excisional skin wounds (13). In mice, topical application of the androgen receptor (AR) antagonist flutamide improves closure of skin wounds (14). A study in men with chronic pressure ulcers failed to show a significant benefit of treatment with the anabolic steroid oxandrolone in a phase 3 trial (15). Studies in male mice with global and selective ablations of *Ar* have yielded a mixture of results reflecting the different strategies used to generate knockouts [reviewed in Chang *et al*. (16)]. Studies in female rodents are rare, but in one study, topical administration of the anabolic androgen stanozolol resulted in increased concentrations of type III collagen and thicker scar tissue in a diabetic mouse model (17). These findings indicate that the role of androgens in wound healing is context dependent and that the potential role in females is poorly understood.

In full-thickness sections of human uterus obtained throughout a normal menstrual cycle [see supplemental figure in Marshall *et al*. (18)], our study revealed intense immunoexpression of AR in stromal fibroblasts in the basal (unshed) region of the endometrium at the time of menses, indicating that they could be a key target for androgen action (18). Using primary human endometrial stromal cells, we have also identified androgen-regulated genes, some of which have been implicated in cell survival, and demonstrated that DHT can delay wound healing in a cell-scratch assay (18). In women with polycystic ovarian syndrome, the ovaries typically produce excess androgens, and some women with polycystic ovarian syndrome report heavy or extended bleeding during menses that would be consistent with an association between unusually high levels of circulating androgens and aberrant endometrial repair (19).

The collected evidence led us to hypothesize that androgens, which are maintained in the blood during menses at a time in the cycle when estrogens and P4 are low, may influence the dynamic changes in cell function that regulate the restoration of endometrial tissue integrity before the onset of the proliferative phase of the normal cycle.

In the current study, we used a mouse model of simulated menses to investigate the impact of androgens on the resolution of a naturally occurring endometrial wound. With this model, we have identified a tightly regulated time frame of endometrial breakdown and repair (7) and changes in expression patterns of genes that are associated with cellular identity, remodeling, and cellular adhesion. In the present study, we describe novel results obtained using this mouse model of menses and speculate that androgens influence both the onset of menses (tissue breakdown) and the time taken to complete scarless repair in women. We believe that these results provide a platform for future studies for exploring ligands that target the AR as novel therapeutics for treatment of disorders associated with disturbances in endometrial repair.

MATERIALS AND METHODS

Mouse model of menstruation and repair

We have described the full protocol for our mouse model of menstruation in a prior publication (7). All mice were ovariectomized to remove endogenous ovarian steroids. In the current study, uterine tissues were recovered only from the uterine horn, in which a robust decidualization response had been induced. On d 19 of the protocol, the mice were split into 3 groups: group 1 (0 h; $n = 11$) had the P4 pellet *in situ* at the time of tissue recovery; group 2 (untreated; $n = 39$) had the P4 pellet removed to simulate P4 withdrawal; group 3 [androgen (DHT) treated; $n = 31$] received a subcutaneous injection of DHT (200 μg/100 μl in oil) simultaneously with P4 pellet removal. Mice in groups 2 and 3 were culled 8, 24, 36, and 48 h after P4 withdrawal. Vaginal smears were taken at each time point to record the presence or absence of blood and therefore evidence of the onset, duration, and cessation of menses. Uteri in all 3 groups were dissected and collected into either RNAlater (Qiagen, Hilden, Germany), or 4% neutral-buffered formalin.

Histology and immunohistochemistry

Samples were processed and subjected to single-color immunocytochemistry according to standard protocols with citrate antigen retrieval (7, 20). The antibodies used were anti-cytokeratin (C2562, mouse monoclonal; 1:100; Sigma-Aldrich, Paisley, United Kingdom), anti-AR (SP107, rabbit monoclonal; 1:100; Spring Biosciences, Pleasanton, CA, USA), anti-matrix metalloproteinase (MMP) 3 (ab52915, rabbit monoclonal; 1:500; Abcam, Cambridge, United Kingdom), anti-MMP9 (sc-10737, rabbit polyclonal; 1:750; Santa Cruz Biotechnology, Dallas, TX, USA), and anti-caspase 3 (9661, rabbit polyclonal 1:400; Cell Signaling Technology, Danvers, MA, USA). Appropriate ImmPRESS horseradish peroxidase Polymer Detection Kits (Vector Laboratories, Burlingame, CA, USA) were used [anti-mouse Ig (rat absorbed) and anti-rabbit Ig], and signals were detected by incubation with 3,3′-diaminobenzidine horseradish peroxidase substrate (Vector Laboratories) and counterstained with hematoxylin and eosin.

RNA extraction

Uterine samples were added to RNeasy Lysis Buffer with 1% 2-mercaptoethanol and homogenized. Lysates were added to RNeasy spin columns, and total RNA was eluted according to the manufacturer's instructions (Qiagen).

Array analysis with the RT2 profiler PCR array

RNA was analyzed with an RNA 6000 Nano kit (Agilent Technologies, Stockport, United Kingdom), according to the manufacturer's protocol: only samples with an RNA integrity number >6.0 were used in the array. RNA samples (400 ng/μl)

were treated to eliminate genomic DNA before preparation of cDNA and analysis using the RT2 profiler Mouse Epithelial-to-Mesenchymal Transition Array (PAMM-090Z; Qiagen, Manchester, United Kingdom) with buffers supplied by the manufacturer; the full list of genes detected by the SYBR Green-optimized primer assays can be found online (*http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-090Z.html*). Analysis was performed as has been described (7), and mRNA concentrations in uterine homogenates prepared from group 2 and 3 mice recovered 24 h after P4 withdrawal were compared to those in group 1. Student's *t* tests determined significance.

Quantitative RT-PCR

Reverse transcription of RNA to cDNA was performed with the Superscript VILO cDNA synthesis kit (Thermo Fisher Scientific Life Sciences, Loughborough, United Kingdom), according to the manufacturer's instructions. RNA samples (100 ng/μl) were incubated at 25°C for 10 min, 42°C for 60 min, and 85°C for 5 min in a thermal cycler. Primers for each gene of interest were designed by the Universal Probe Library Assay Design Center (Roche Applied Science, Penzberg, Germany) and purchased from Eurofins MWG Operon (Ebersberg, Gemany). (Sequences are shown in Supplemental Table 1.) Reactions were prepared in duplicate. Amplification was performed at 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Analysis was performed by using the $\Delta\Delta C_t$ method, where each sample was expressed relative to the endogenous control *18s*. For all PCR analyses, all samples were compared to the 0 h sample set (group 1), according to the array, and values were plotted as fold changes ± SEM.

Chromatin immunoprecipitation

Putative androgen response elements (AREs) within 500 bp of the transcription start site of genes of interest were identified by using the Mapper database (21). With these putative sites as a guide, we designed 2 sets of primers for each gene using ENSEMBL (*www.ensembl.org/index.html*) and Primer3 (*http://primer3.ut.ee/*). Primers were purchased from Eurofins MWG Operon. (See Supplemental Table 1 for sequences.)

Chromatin extraction was performed using the truChIP Chromatin Shearing Kit (Covaris, Woburn, MA, USA), per the manufacturer's instructions. Uteri from 4 untreated wild-type C57Bl/6J mice were pooled so that the optimal tissue size of 80 mg could be used. Samples were sonicated at 4°C on a Bioruptor (Diagenode, Seraing, Belgium) to shear the chromatin to 200–1000 bp. Sheared supernatants were carried forward to chromatin immunoprecipitation (ChIP), which was performed with the MAGnify Chromatin Immunoprecipitation System (Thermo Fisher Scientific Life Sciences), according to the manufacturer's instructions. In brief, a ChIP-grade AR antibody (ab74272; Abcam) was coupled to Dynabeads at 5 μg/ml for 1 h, and positive (RNA pol II) and negative antibody controls (39097; Active Motif, Carlsbad, CA, USA) were included at 2 and 1 μg/ml, respectively. Input controls for each sample were included. Chromatin was bound to the beads at 4°C for 24 h before they were washed. Reverse cross-linking was performed at 55°C for 15 min, and the DNA was purified, washed, and eluted. Extraction of DNA for all samples was confirmed by PCR analysis with a known endogenous control primer set, EF1 (53011).

PCR analysis for genes of interest was performed with standard PCR techniques, where amplification was continued for 40 cycles at 94°C for 20 s, 59°C for 30 s, and 72°C for 30 s. For every gene of interest, an input control, positive control, and negative control sample were included. PCR products were analyzed with Qiaxcel (Qiagen).

RESULTS

Treatment with DHT alters the onset and duration of vaginal bleeding

In agreement with results from women (18) stromal cells in the uterus of intact and ovariectomized mice express AR with intense immunopositive staining in the nuclei of stromal fibroblasts (22). Thus, the impact of DHT was investigated.

In untreated (group 2) female mice (**Fig. 1*A***), vaginal bleeding (a surrogate for tissue breakdown/shedding) was detected within 4 h of P4 withdrawal in 5 of 13 animals (~40%,), and overt bleeding had stopped in all mice by the 24-h time point. In contrast, group 3, DHT-treated females, had a slower onset and longer duration of bleeding. Although only 1 androgen-treated mouse (17%) had blood cells in the vagina at the 4-h time point, the proportion had risen to 100% ($n = 10$) at the 24-h time point, and this was also the case in 3 of 5 animals at 36 h. No blood was detected at 48 h, suggesting that endometrial healing was complete. Injection of DHT also had a significant impact on the uterine wet weight of the decidualized horn at the 24- and 36-h time points when compared with tissues recovered from group 2 animals, consistent with a delay in endometrial shedding and breakdown (Fig. 1*B*).

Histologic evaluation revealed that androgen supplementation had an impact on both the onset of breakdown and the restoration of epithelial integrity

Endometrial tissue collected from group 1 females (**Fig. 2*A*, *B***, 0 h) exhibited a robust decidualization response characterized by the presence of large nuclei in the stromal cell mass (inset, higher magnification). In line with expectations, 8 h after removal of the P4 pellets from group 2 mice (Fig. 2*E*, *F*), the decidualized tissue mass had detached from the underlying basal layer, leaving the stromal compartment exposed and without an overlying epithelium (no layer of cytokeratin-positive epithelial cells; brown stain). In contrast, in group 3 (DHT-treated) females, the decidual mass had not detached from the basal layer, and there was an intact cytokeratin-positive epithelium lining the lumen (Fig. 2*G*, *H*). However, in these females, there were detectable erythrocytes in the decidual cell mass, which was consistent with the initiation of breakdown and coincided with detection of blood in the vagina at that time.

At the 24-h time point, a striking difference between uterine tissue architecture in group 2 and 3 females was detected. In group 2 females (Fig. 2*I*, *J*), the decidual cell mass was detached from the underlying stroma, but some cytokeratin-positive cells were detected adjacent to the lumen (Fig. 2*J*). In the group 3 animals (Fig. 2*K*, *L*), the decidualized mass had broken down, but detachment from the basal compartment was incomplete (Fig. 2*K*), consistent with a delay in breakdown and shedding. Cells immunopositive for pan cytokeratin were also detected in the basal layer of the tissue, but were restricted to small

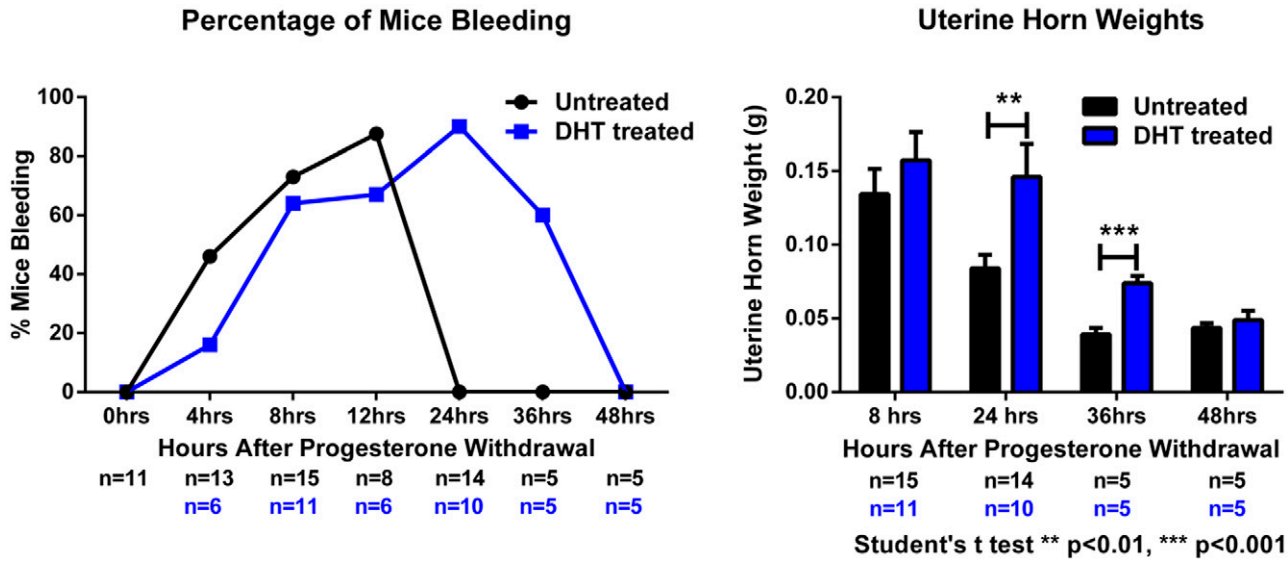

Figure 1. Treatment with DHT has a striking impact on onset and duration of vaginal bleeding. Bleeding observations and uterine horn weights were recorded. *A*) The percentage of mice with blood in the vagina between 0 and 48 h after the withdrawal of P4 was calculated as a percentage of mice that were identified to be bleeding at each time point of the total number of mice examined at each time point. Note that some data from the group 2 females (black line, 0–24 h) are the same as those shown in an earlier study (7) but the data for 36 and 48 h are new. Group 3, DHT-treated mice are indicated by the blue line. The number of animals in each group is given in the appropriate colors below the time points. *B*) Upon dissection, uterine horns were cleaned and stripped of fat and each horn weight recorded. Weights are displayed as mean grams ± SEM. Student's *t* test was performed to determine any significance between the untreated and treated animals for each time point. Significance was accepted as $P < 0.05$.

areas (Fig. 2*L*). No residual luminal epithelium was present, which, based on our previous findings (7), may have contributed to a delay in repair.

At the 36-h time point, the luminal epithelium in group 2 animals (Fig. 2*M*, *N*) was intact, denoted by a continuous layer of cytokeratin-positive epithelial cells, consistent with completion of epithelial repair. In contrast, in group 3 animals (Fig. 2*O*, *P*), remnants of the functional decidua failed to detach from the underlying stroma and the luminal compartment did not fully re-epithelialize (Fig. 2*P*). By 48 h, an intact luminal epithelium was identified in all group 2 females, with some decidual debris remaining in the lumen (Fig. 2*Q*, *R*). An intact cytokeratin-positive luminal epithelium was also present in group 3 females (Fig. 2*S*, *T*); however, it was not uniform, and the underlying stroma appeared still to be undergoing some remodeling (Fig. 2*T*).

Array analysis identified androgen-dependent changes in mRNA concentrations of genes associated with cellular adhesion and remodeling

In earlier work, we identified dynamic changes in concentrations of mRNAs encoded by key genes associated with the process of mesenchymal–epithelial transition during the active period of shedding and repair in our mouse menses model (7). In the current study, uteri of mice from groups 2 and 3 recovered at the 24-h time point were analyzed with a PCR array to complement and extend the data on bleeding status, uterine wet weight, and morphology. Treatment with DHT generally reduced the concentrations of mRNAs detected by the array (Supplemental Table 2). Five genes—*Mmp3*, *Mmp9*, *Snai3*, *Ocln*, and *Spp1*—that were up-regulated by greater than 10-fold in uterine samples recovered from group 2 mice relative to those in group 1 were chosen for further investigation, because in each case the array data suggested a striking reduction in the concentrations of their mRNAs in the uteri of mice in group 3 (**Table 1**). Validation by qRT-PCR with additional samples and new sets of primers (**Fig. 3**) demonstrated a significant difference in mRNA concentrations for *Mmp3*, *Mmp9*, *Snai3*, and *Spp1* in DHT-treated animals at the 24-h time point. The difference in the concentration of *Ocln* mRNA was not statistically significant. Additional analysis across all of the time points highlighted temporal regulation in both groups 2 and 3 (Supplemental Fig. 1) and revealed that, although uterine wet weights and morphology were similar in both groups at the 48-h time point (Figs. 1*B* and 2*Q*, *T*), mRNA concentrations remained significantly different for *Mmp9*, *Spp1*, and *Ocln* ($P < 0.05$, $n = 5$/group), consistent with extensive remodeling in the DHT-treated tissue (Supplemental Fig. 1).

Presence of a putative ARE in genes identified by array analysis was consistent with direct regulation of gene expression by ligand-bound AR

The selected genes identified as androgen regulated by the epithelial–mesenchymal transition array were further investigated for the presence of an ARE

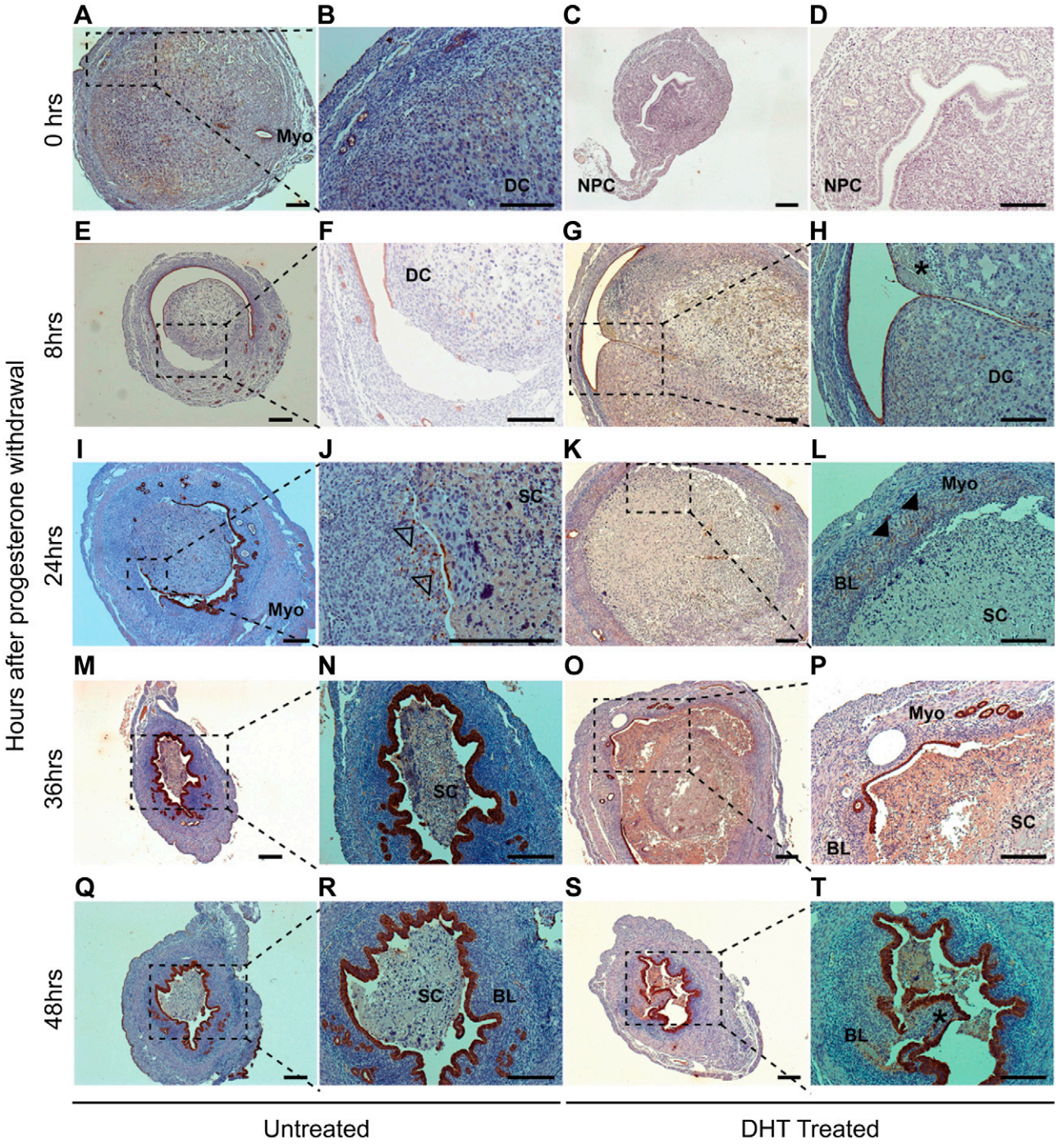

Figure 2. Uterine morphology during breakdown and repair is altered with DHT treatment. Immunohistochemistry for pan-cytokeratin (brown) highlights epithelial cell dynamics across menses. *A, B*) At the time of P4 withdrawal, glandular epithelial cells in the basal layer were positive for cytokeratin. *C, D*) Negative primary antibody controls (NPC). *E–H*) At 8 h, the functional stroma has detached from the underlying basal layer in untreated animals but not treated animals. Evidence of red blood cells is denoted by an asterisk (*H*). *I–L*) New epithelial cells lined the luminal surface in untreated animals at 24 h (*J*, open arrowheads), whereas, in DHT-treated animals, cytokeratin-positive cells were located in the stroma (*L*, solid arrowheads). *M, N*) At the 36-h time point, the luminal epithelium in untreated animals was intact. *O, P*) In DHT-treated animals, remnants of the functional decidua had failed to detach from the underlying stroma, and the luminal compartment (*P*) had not fully reepithelialized. *Q, R*) Untreated animals were repaired by the 48-h time point. *S, T*) DHT-treated animals were still undergoing remodeling at the 48-h time point (asterisk denotes stromal remodeling) ($n = 3$ animals per time point). Dashed boxes enclose sections shown at higher magnification in the following images. DCs, decidual cells; SC, shed cells; BL, basal layer; Myo, myometrium. Scale bars, 200 μm.

upstream of their promoter using the MAPPER database (21). Putative AREs were then analyzed by ChIP of tissue prepared from uterine horns of intact cycling mice and an antibody specific for AR. Of the 5 genes investigated, 3—*Mmp3*, *Snai3*, and *Spp1*—were identified by MAPPER as having putative AREs, which were subsequently confirmed by ChIP (Table 2 and Supplemental Fig. 2).

TABLE 1. *Summary of genes identified using the RT2 array chosen for validation*

Gene	Fold regulation	
	24-h untreated	24-h–DHT treated
Mmp3	61.2906	9.8823
Mmp9	28.7339	6.8393
Snai3	15.8243	0.0
Spp1	23.2021	13.5539
Ocln	11.2243	2.5191

Values are normalized to group 1, 0-h time point.

Androgen treatment resulted in altered immunoexpression of MMP9 and -P3, as well as the apoptosis marker cleaved caspase 3

In women, MMPs are known to play a critical role in endometrial breakdown and repair during menses, controlling the shedding of the functional layer and remodeling of the tissue (23–25). In this study of a mouse model of simulated endometrial breakdown and repair, injection of DHT resulted in significant changes in concentrations of mRNAs encoded by *Mmp3* and *Mmp9* in uterine homogenates (Fig. 3). However, because expression of MMP proteins can occur *via* transcriptional, posttranscriptional, or post-translational mechanisms immunolocalization for MMP3 and -9 was undertaken.

Immunostaining for MMP9 revealed both temporal and spatial expression, with staining less intense at the 24-h time point than at 48 h in both groups 2 and 3 (**Fig. 4*A*** *vs.* Fig. 4*C* and Fig. 4*B* *vs.* Fig. 4*D*). At the 48-h time point, intense immunoexpression of MMP9 was detected in the luminal epithelium and adjacent stromal cells, as well as in the glandular epithelium in both groups (Fig. 4*C*, *D*).

At the 24-h time point in the uteri of group 2 females, intense immunostaining for MMP3 was localized to areas of exposed basal stroma, consistent with active breakdown and remodeling of the tissue (Fig. 4*E*), with weak or no staining in areas where the epithelium was intact (Fig. 4*E*, inset). In group 3 mice recovered at the same time point, the decidualized functional layer had not shed, and immunopositive staining for MMP3 was detected throughout the decidualized mass (Fig. 4*F*), but was only as intense as in group 2 mice in the basal functional zone adjacent to the region where tissue shedding was initiated (Fig. 4*F*, inset). In both groups 2 and 3, in uterine tissue recovered 48 h after P4 withdrawal, intense immunopositive staining for MMP3 was detected in epithelial cells of the newly restored luminal epithelium, with some residual staining in the underlying stromal compartment in the group 3 samples (Fig. 4*G*, *H*, insets).

Immunostaining for cleaved caspase 3 revealed a striking parallel with the pattern of expression of MMP3 at 24 h (Fig. 4*I*, *J* *vs.* Fig. 4*E*, *F*), suggesting apoptosis in association with tissue remodeling at this time point. Notably, caspase 3 staining was localized to areas of exposed basal stroma in group 2 and at regions of tissue shedding in group 3. By the 48-h time point, in both groups 2 and 3 (Fig. 4*K*, *L*, respectively), caspase immunostaining

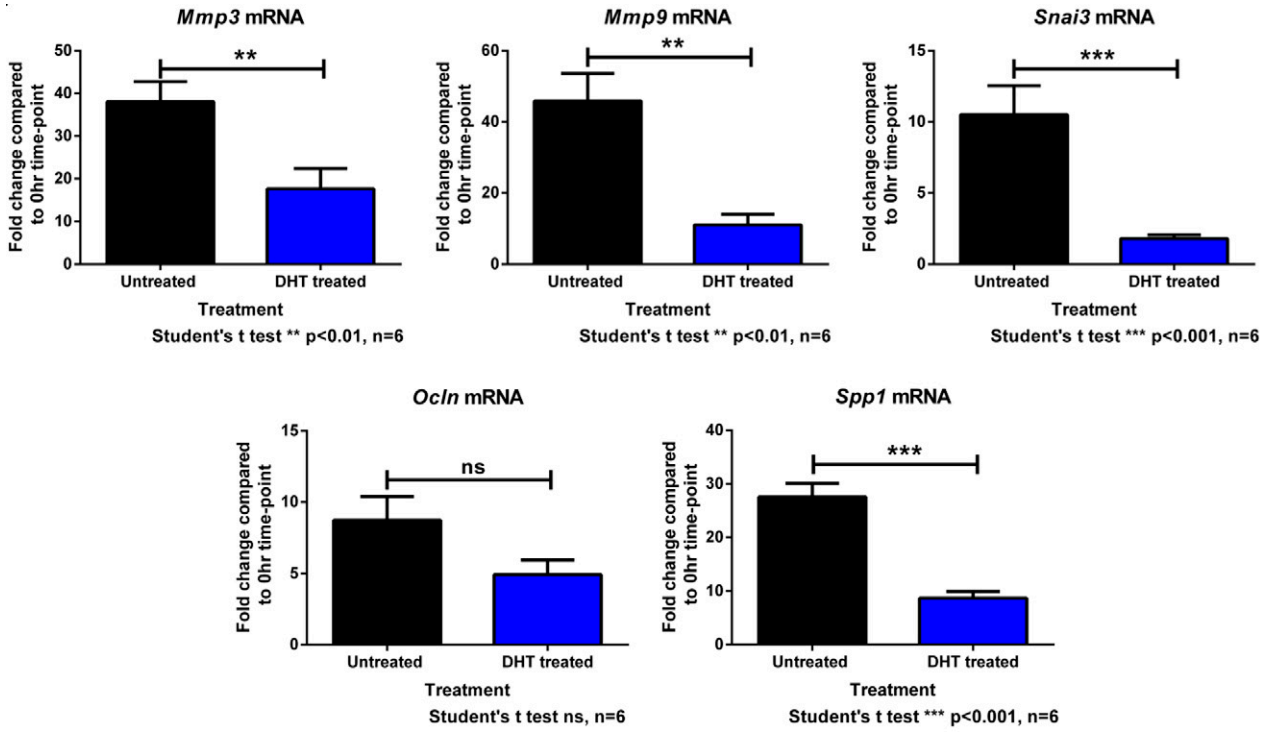

Figure 3. Genes associated with tissue remodeling, cell adhesion, and cell matrix are altered by DHT treatment. Validation of androgen-regulated genes using samples recovered at 24 h, with or without DHT ($n = 6$). All samples were normalized against the endogenous control *18s* and then compared against group 1, per the PCR array. Significance was set at $P < 0.05$.

TABLE 2. *Presence of putative AR binding sites within gene promoters*

Gene	Putative ARE[a]	ARE confirmed by ChIP
Mmp3	1	Yes
Mmp9	0	N/A
Snai3	1	Yes
Spp1	1	Yes
Ocln	0	N/A

N/A, not applicable. [a]Within 500 bp of the promoter.

was limited to just a few cells in the luminal epithelium; the underlying stroma was immunonegative. On the basis of this evidence, it appears that the delay in tissue breakdown and remodeling that was observed in group 3 females was paralleled by the timing of active cell death, as indicated by immunopositive staining for caspase 3.

DISCUSSION

The endometrium is a remarkable tissue that may experience up to 400 episodes of breakdown and repair over the course of a woman's reproductive lifespan. What is more remarkable is its ability to repair, without scarring, over just a few days. The endometrium is one of only a few adult tissues that heal without formation of a scar. Studies on wound healing in other tissue sites have reported that oral mucosal wounds display significantly less clinical and histologic scar formation than corresponding skin wounds (26). Scarless repair is also a feature of fetal tissues, and research has been directed at understanding why fetal skin does not scar, in the hope that such studies might help develop a novel strategy to improve treatment for patients at risk of excessive scar formation (27), and a greater understanding of the regulation of endometrial repair might also help inform this goal.

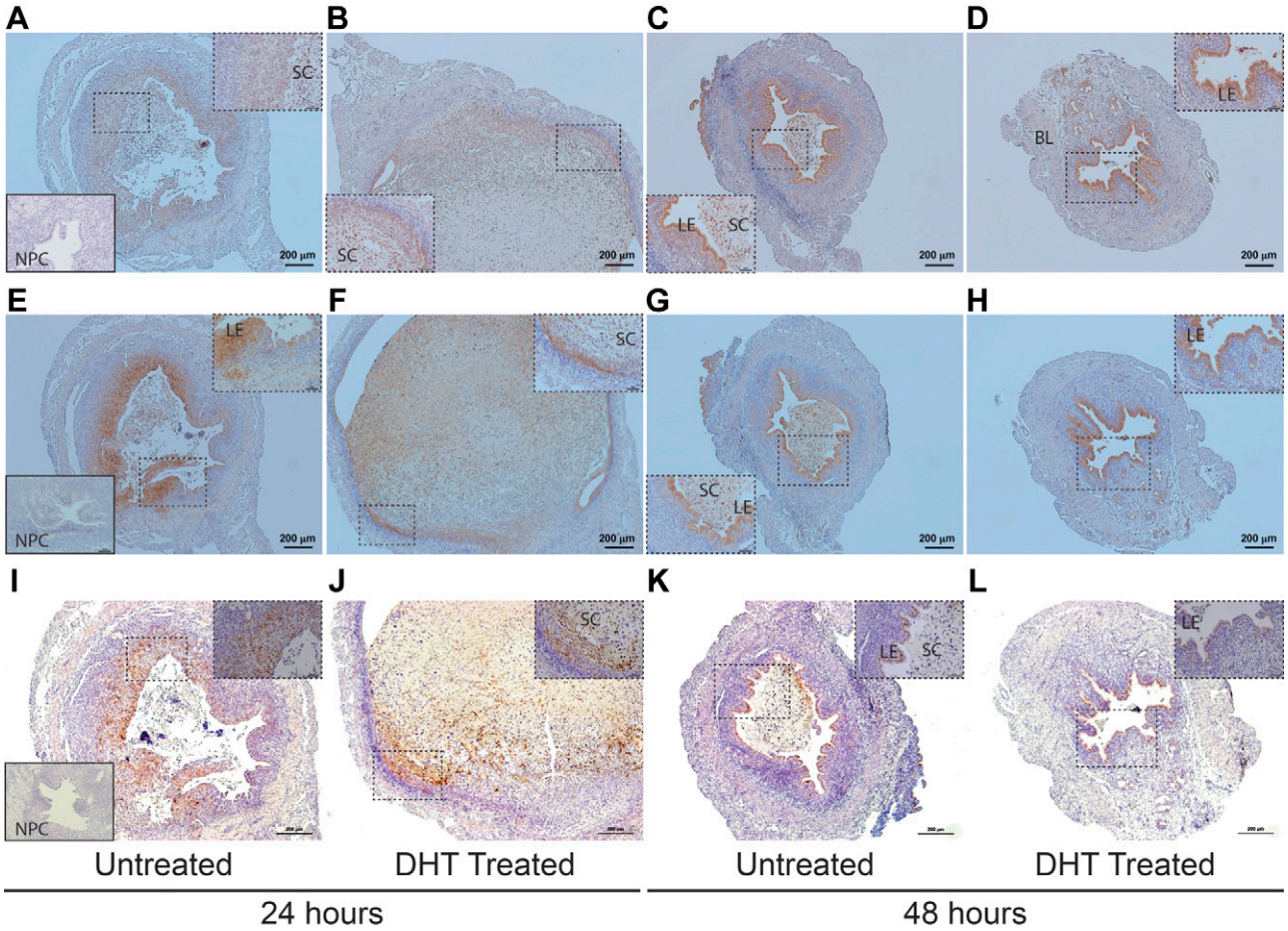

Figure 4. Temporal and spatial immunolocalization of MMP9, MMP3, and Caspase 3 was observed during repair and remodeling. Cross sections of uteri from groups 2 (*A, C, E, G, I, K*) and 3 (*B, D, F, H, J, L*) recovered 24 or 48 h after P4 withdrawal are shown. *A–D*) Immunostaining for MMP9 was of low intensity and largely confined to stromal cells after 24 h (*A, B*), but with a striking increase in both intensity and pattern at 48 h, with strong staining of epithelial cells that lined the newly restored luminal and glandular compartments (*C, D*). *E*) In group 2 mice, at 24 h, MMP3 was localized to regions of the basal stroma that were still undergoing reepithelialization (inset). *F*) In contrast, in group 3 mice, MMP3 protein was most abundant in the stromal cells at the junction of the shedding functional layer (inset). *G, H*). At the 48-h time point, in both groups 2 (*G*) and 3 (*H*), MMP3 was immunolocalized to the luminal epithelium. *I–L*) Spatial and temporal immunoexpression of caspase 3 largely paralleled that of MMP3 with differential expression between group 2 (*I*) and 3 (*J*) samples at 24 h and most prominent staining in the epithelial compartment at 48 h (*K, L*) NPC; negative primary control. LE; luminal epithelium, SC; shed cells. Scale bars, 200 μm; insets, 50 μm.

Imaging of human endometrium during menstruation has revealed that endometrial shedding and restoration of an intact luminal epithelium occurs simultaneously, and this process is thought to ensure rapid recovery of tissue homeostasis and limit blood and fluid loss (4). Effective endometrial repair relies on coordinated, and tightly regulated, breakdown and tissue remodeling, to ensure restoration of tissue integrity. Dysregulation of repair mechanisms is thought to underlie the development of several conditions, including oral cavity fibrosis, Asherman's syndrome (intrauterine fibrosis) and heavy menstrual bleeding. Although the etiology of oral cavity fibrosis is unclear, changes in the extracellular matrix are thought to be caused by increased collagen production and inflammation (28, 29). Asherman's syndrome is an acquired condition caused by damage to the endometrium that has typically been associated with endometrial curettage or pelvic surgery (30, 31), but may also occur in 20% of women after miscarriage (32). Recent studies have reported increased expression of fibrotic markers, including collagens I and III, in endometrial tissue adhesions (33). Novel therapeutic approaches to treat the disorder, which have been modeled in rodents, but not yet applied in patients, include transplantation of bone marrow–derived stem cells (34) and oral mucosal cell sheets (35).

We, and others, have used mice with induced menstruation (7–9) or xenografting of human tissue fragments into immunocompromised mice (10) to interrogate the processes that contribute to repair at the time of the menses. We have focused our investigations within a 48 h repair window. We have recently shown, in an ovariectomized mouse model, that treatment with DHT can regulate uterine function, but does not alter estrogen receptor-α expression in the endometrium (22), supporting the data in the current study that androgens can act independent of estrogens.

Androgenic regulation of endometrial repair has not been considered, although it is notable that women have significant concentrations of circulating androgens in their bloodstream during the menstrual phase of the cycle (1, 36). Androgens have been implicated, however, in the regulation of endometrial receptivity and establishment of pregnancy with evidence from *in vitro* studies that decidualizing endometrial stromal cells acquire the ability to synthesize and secrete both T and DHT (37). Exogenous addition of androgens to human decidualized stromal cells appeared to protect the cells from oxidative stress (38), suggesting that local increases in bioavailable androgens within the tissue that are not detected by measuring circulating concentrations of steroids also play a role in protecting the tissue at the onset of menses in women. Although the enzymes necessary for intrauterine biosynthesis of androgens are also present in the mouse uterus (39, 40), mice do not synthesize adrenal androgens that form the key precursor pool in women. Thus, in the current study, we used an exogenous dose of DHT to explore whether an AR agonist that cannot be metabolized to estrogens could have an impact on endometrial tissue breakdown in a well-defined model. In their 1996 paper, Zhang and Croy (41) explored the relative impacts of androgens (T, DHT) and P4 on maintenance of an artificially induced decidualization response in mice. They showed that multiple injections of androgens (1 or 2 mg/mouse) maintain uterine wet weights in the absence of P4 for up to 4 d, but they did not examine tissue after withdrawal of P4, T, or DHT. In the current study, we used a 5-fold lower dose of DHT but also observed maintenance of uterine wet weight after a single dose of DHT, consistent with delayed tissue breakdown.

Cutaneous wound healing shares several features with endometrial wound repair at the time of menses, including infiltration of immune cells, tissue remodeling, and reepithelialization (42, 43). Studies in aging men and women have revealed differences in the rates of cutaneous wound healing consistent with a role for sex hormones in regulating these processes. Furthermore, AR has been detected in multiple cell types in skin, including keratinocytes, dermal fibroblasts, and macrophages. Studies in male mice (high endogenous androgens) with selective ablation of *Ar* suggest that keratinocyte AR promotes reepithelialization, whereas fibroblast AR suppresses it (44). Mice that have an inactivating mutation in their *Ar* gene (Tfm; testicular feminization) have decreased collagen content in the skin (45).

A recent study of human endometrial stromal cells reported that activation of AR is associated with changes in genes that are involved in regulation of the cytoskeleton (46). In our own studies, treatment of primary human endometrial stromal cells with DHT reduced the rate of cell migration, as determined with an *in vitro* wound-healing (scratch) assay (18). Results in the current study would also be consistent with DHT acting *via* AR in stromal cells and thus having an impact on the rate of wound repair. The speculation that androgens, either from the circulation or synthesized in endometrial tissue in association with decidualization, *modulate* repair of the menstrual appear to fit with findings in males where blocking biosynthesis of DHT using a 5α reductase inhibitor *accelerates* wound closure (47).

MMPs are well established as playing a critical role in endometrial breakdown and repair (23, 48), and it is notable that both MMP3 and -9 were differentially regulated by androgen treatment in our model. MMP3 and -9 have been identified as key enzymes involved in wound healing and tissue repair in the skin (49), and androgens have been reported to alter the expression of matrix proteins during cutaneous wound healing (50). Castration of male rats resulted in decreased expression of collagenases MMP1 and -13 and gelatinases MMP2 and -9, with greater collagen deposition at wound sites. Conversely, in the current study, treatment with androgens resulted in decreased concentrations of MMP9, suggesting gender or tissue-specific differences in regulation by androgens. Steroidal regulation of MMPs is not a new concept; P4-driven inhibition of MMP9 was documented over 20 yr ago (51). However, more recent studies in the baboon have demonstrated that, whereas MMP7 is negatively regulated by P4, MMP3 is regulated by a different mechanism (52). In the current study, *Mmp3* was identified to contain an ARE in its proximal promoter region and is therefore a good candidate for being a direct target for AR-dependent gene regulation. In contrast, no ARE was identified within 1000 bp upstream of the promoter of *Mmp9*, even though we documented a robust and significant reduction in

concentrations of its mRNA in total tissue homogenates of the uterus of mice treated with DHT. MMP3 is a known activator of MMP9 (53, 54), and it is therefore possible that DHT indirectly regulates MMP9 through MMP3 and its cleavage of proMMP9. In this study, there were also parallels between the temporal–spatial immunoexpression of MMP3 and caspase 3, providing a link between androgen-dependent gene expression and apoptosis.

The data generated by the current mouse model suggest that androgens in the blood bathing the human endometrium during menses could modulate the rate and extent of breakdown of the endometrium through both direct and indirect regulation of MMPs (and cell adhesion molecules) to control tissue destruction and avoid excess shedding. Based on our own studies (18), we believe androgens may also reduce apoptosis of basal endometrial fibroblasts so that they can contribute to restoration of endometrial tissue integrity by providing both the platform for epithelial migration and by undergoing mesenchymal–epithelial transition (7, 55).

The recent development of nonsteroidal selective AR modulators offers an attractive therapeutic alternative to treatment of women with bioactive androgens, such as DHT, which can have unwanted virilizing side effects (56). Selective AR modulators have already passed the necessary checks for human trials, have been tested in women for muscle loss associated with cancer (57), and have been shown to improve bone density and reduce osteoporosis in both men and women (58).

CONCLUSIONS

This study of a mouse model of menses explored the role of androgens in scarless repair of the endometrium and provides the first evidence that AR-dependent regulation of MMPs may contribute to regulation of wound healing in this tissue, demonstrating parallels with cutaneous wound healing.

The study is the first to consider a role for androgens and the AR during menstruation in modulating the pace of endometrial shedding and repair, and we speculate that the results obtained suggest that drugs targeting the AR may be used for the development of treatments for endometrial disorders that affect many millions of women. FJ

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